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High intensity exercise decreases IP6K1 muscle content & improves insulin sensitivity (S_I^{2*}) in glucose intolerant individuals

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Context:

Insulin resistance in skeletal muscle contributes to whole body hyperglycaemia and the secondary complications associated with type 2 diabetes. Inositol hexakisphosphate kinase-1 (IP6K1) may inhibit insulin-stimulated glucose transport in this tissue type.

Objective:

Muscle and plasma IP6K1 were correlated with two-compartment models of glucose control in insulin-resistant hyperinsulinemic individuals. Muscle IP6K1 was also compared following two different exercise trials.

Methods:

Nine pre-diabetic [HbA1c; 6.1 (0.2) %] were recruited to take part in a resting control, a continuous exercise (90% of lactate threshold) and a high-intensity exercise trial (6 x 30 sec sprints). Muscle biopsies were drawn pre- and post each 60-minute trial. A labeled ([6,6²H₂]glucose) intravenous glucose tolerance test (IVGTT) was performed immediately after the second muscle sample.

Results:

Fasting muscle IP6K1 content did not correlate with S_I^{2*} ($P = 0.961$). High-intensity exercise reduced IP6K1 muscle protein and mRNA expression ($P = 0.001$). There was no effect on protein IP6K1 content following continuous exercise. Akt³⁰⁸ phosphorylation of was significantly greater following high-intensity exercise. Intermittent exercise reduced hepatic glucose production (HGP) following the same trial. The same intervention also improved S_I^{2*} and this was significantly greater compared to the continuous exercise improvements. Our *in vitro* experiment demonstrated that the chemical inhibition of IP6K1 increased insulin signaling in C2C12 myotubes.

Conclusions:

The *in vivo* and *in vitro* approaches used in the current study suggest that a decrease in muscle IP6K1 may be linked to whole body improvements in S_I^{2*} . In addition, high-intensity exercise reduces HPG in insulin-resistant individuals.

This work investigated the role IP6K1 plays in causing insulin resistance (IR) and found that high-intensity exercise reduces IP6K1, improves IR & hepatic glucose production in hyperinsulinemic humans.

Background

Type 2 diabetes (T2Ds) is a multifactorial metabolic disease characterized by defects in insulin sensitivity (S_I), glucose effectiveness (S_G), β -cell function and endogenous glucose production (EGP) (1). Although not conclusive, insulin resistance seems to occur due to a decrease in the insulin receptor substrate's (IRS) ability to activate downstream insulin signaling kinases (2). A reduction in the serine/threonine protein kinase Akt phosphorylation is a known characteristic of insulin resistance and type 2 diabetes (3) and is an important protein in the insulin-signaling cascade.

Insulin-stimulated glucose uptake involves insulin receptor autophosphorylation, tyrosine phosphorylation of insulin receptor substrate (IRS), and the subsequent activation of phosphatidylinositol (PI) 3-kinase. (PI3K). The downstream target of PI3K, Akt is then

activated via the phosphorylation of Thr³⁰⁸ and Ser⁴⁷³ by phosphatidylinositol 3,4,5-triphosphate-dependent protein kinase (PDK)-1 & PDK-2, respectively (4). Akt contains an N-terminal pleckstrin homology (PH) domain, allowing for the binding of phosphatidylinositol-3,4,5-triphosphate (PIP3), and the subsequent membrane translocation & subsequent activation of Akt (5). Upon activation, Akt is then responsible for the subsequent phosphorylation of AS160, GLUT4 translocation, glucose uptake (6) phosphorylation and inhibition of GSK3 β & glycogen synthesis (7, 8); making Akt a potential target in the treatment of type 2 diabetes.

Diphosphoinositol polyphosphates, also known as inositol pyrophosphates, are a family of water-soluble inositol phosphates (9). The inositol hexakisphosphate (IP6) kinase 1 (IP6K1) produces a pyrophosphate group at the 5th position of IP6 to generate a further inositol pyrophosphate, diphosphoinositol pentakisphosphate (5-PP-1(1,2,3,4,6)IP5 or IP7) (10). Production of IP7 results in its binding to the PH domain of Akt/PKB, preventing its translocation to the cell membrane and reducing its subsequent phosphorylation by PDK1. The evidence for this comes from the finding that IP7 fails to prevent PDK1 phosphorylation of Akt^{Thr308} lacking a PH domain (11). The consequence of this is a potential reduction in insulin (Akt)-stimulated glucose uptake in muscle and adipose tissue (12). (11) have shown that IP6K1 KO mice demonstrate augmented Akt activity and increased glucose transport rates in skeletal muscle. Key to the current work, recent *in vivo* data suggests a novel role of IP6K1 in insulin resistance with IP6K1 KO mice displaying normal glycaemic control despite low circulating plasma insulin (13). In addition, the pharmacological inhibition of IP6K1 increases Akt signaling in mouse embryonic fibroblasts (MEF), while suppressing IP7 synthesis (14). The increased availability of IP6K1 and its product IP7, are thought to be stimulated by insulin. The competition of IP7 with PIP3 for binding at PH domain of Akt may represent a negative feedback mechanism whereby hyperinsulinaemia eventually decreases Akt activity, indirectly shown through the presence of decreased insulin action despite augmented insulin secretion in pre-diabetic states (15) and an increase in IP6K1 activity and reduced p-Akt in rodents treated with insulin (11).

However, research suggesting a role of IP6K1 inhibition as a future target of insulin resistance has been limited to *in vitro* and animal work. The current study aimed to tackle this by measuring IP6K1 muscle content in hyperinsulinaemic insulin resistant humans.

Insulin-stimulated glucose uptake increases from 2 to 72 hours post exercise (16, 17, 18) with the amount of muscle mass an important determinant to this response, thus exercise involving a larger muscle mass is preferable. Higher intensity exercise recruits a larger proportion of muscle as well as a greater number of type 2 glycolytic muscle fibres compared with moderate-intensity activity (19), which may offer a greater *sink* for glucose disposal. Recently a form of high-intensity interval training (HIIT) has been shown to improve S_I (20, 21, 22). However, data on the other metabolic defects associated with type 2 diabetes, including hepatic glucose production, β -cell function and glucose effectiveness, remains sparse. Here we assessed the effects of high-intensity exercise on two-compartment models of insulin sensitivity, glucose effectiveness and hepatic glucose production.

Therefore, the aims of this study were to firstly characterise this novel insulin signalling pathway (Akt-IP6K1) in pre-diabetic humans for the first time. Secondly, using a stimulus known to improve insulin sensitivity (muscle contraction), we aimed to examine if IP6K1 could be manipulated following muscle contraction in glucose intolerant individuals and to evaluate the effects of different types of exercise stimuli on IP6K1 muscle content. The C2C12 skeletal muscle cell line was also used to investigate the role of N2-(m-Trifluorobenzyl), N6-(p-nitrobenzyl)purine (TNP), treatment on Akt-AS160 signalling.

Participants and ethics

In vivo study - nine sedentary glucose intolerant individuals (7 male & 2 female) were recruited for this investigation. Subjects' clinical characteristics were age, 47 (3) yr; BMI, 32.0 (2.4); Body Fat, 39.0 (4.4); HbA_{1c} 6.1 (0.2); HOMA_{IR} 3.3 (0.8). Each participant was informed of the study purpose, experimental procedures, and all of its potential risks prior to providing written consent to participate. Ethical approval was granted by the local University Ethics Committee (Ref: 11_12_23_) and conformed to Declaration of Helsinki for the use of human participants in research. Exclusion criteria included diabetic-related complications (i.e. neuropathy, peripheral vascular and cardiovascular disease), current smokers or treatment with insulin or any other pharmaceutical intervention. Glycated haemoglobin (HbA_{1c}) values of >5.7% and <6.4% were used to define individuals in a pre-diabetic state (23).

Experimental protocol

Participants were required to attend our laboratory on four occasions each separated by 7-14 days. During a preliminary visit percentage of body fat was estimated using Bodpod as previously described (24). Venous blood samples were drawn for the determination of HbA_{1c} (Axis-Shields, U.K.). Fasting blood glucose and plasma insulin concentrations were measured for the determination of homeostasis model of insulin resistance [HOMA_{IR}; fasting insulin (μU/ml) x fasting glucose (mmol/l) / 22.5] and HOMA of β-cell function [HOMA_{β-cell}; 20 x fasting insulin (μU/ml) / fasting glucose – 3.5 (mmol/l)] (25). During this preliminary visit, individual lactate threshold (LT) values were obtained as previously described (26) using a cycle ergometer (Lode Corival).

On experimental days, volunteers reported to the laboratory at ~08:30 having fasted for 12 hr, abstained from caffeine and alcohol for 24 hr, and exhaustive exercise for 72 hr. An 18-gauge cannula was positioned into a dorsal hand vein to allow for frequent sampling of arterialised blood, using a thermoregulated hot box (~60°C) (27). A second 18-gauge cannula was placed into a prominent contralateral antecubital vein for administration of labelled glucose. In a randomised fashion, subjects completed 1) a resting control trial (Rest) of 60 minutes of passive sitting, 2) continuous exercise at an intensity equal to 90% LT for 60 minutes (Continuous) (cycle ergometer, Lode Corival) and 3) high-intensity intermittent exercise (6 x 30 sec Wingate's) (Intermittent) (Monark 894 E, Weight Ergometer). The 30 sec sprints were interspersed with 9.5 minutes of passive recovery in the intermittent trial. Each trial lasted 60 minutes in duration. Muscle biopsies were drawn under local anesthesia from vastus lateralis using the conchotome method (28) at baseline (0 min) and immediately post (60 min) trials with volunteers in a supine position. Immediately after the post-treatment muscle biopsy, a 4 hour labelled intravenous glucose tolerance test (IVGTT) was administered (28.4 mg/kg of [6,6²H₂]glucose & ~250 mg/kg of unlabeled glucose), prepared under sterile conditions. Thereafter, frequent arterialized (~5 mL) blood samples were drawn over the ensuing 240 min, as previously described (26). The concentration of circulating glucose was measured in whole blood using a YSI 2300 (STAT; Yellow Springs, USA), whilst spun separated (4 °C, 10 minutes, 5 000 rpm) plasma was frozen and later analyzed for plasma insulin, endogenous glucose and isotope enriched [6,6²H₂]glucose concentrations.

Blood Analysis

Glucose enriched plasma samples were deproteinized in ethanol (99%) with the resulting supernatants centrifuged to dryness. Hydroxylamine hydrochloride (100 μl, 0.18 M pyridine) was then added before a 60 minutes incubation at 70 °C. After which, Bis(trimethyl)trifluoroacetamide: 1% trimethyl- chlorosilane (TMCS; 99%) (Sigma-Aldrich, Exeter, UK) before a further incubation (45 min at 70 °C). Samples were then analysed for

glucose derivatives of 319 (unlabeled glucose; trace) and 321 ($[6,6^2\text{H}_2]$ glucose; tracer) by GC/MS. Endogenous glucose concentration was measured in whole blood (YSI 2300; STAT; Yellow Springs, USA) and plasma insulin using a commercially available ELISA (DRG diagnostics, UK).

Plasma insulin, endogenous glucose concentrations and $[6,6^2\text{H}_2]$ glucose-enriched values were used to model the metabolic indices; insulin sensitivity (S_I^{2*}), glucose effectiveness (S_G^{2*}), hepatic glucose production (HGP), acute insulin response to glucose (AIR_g) and disposition index ($DI = S_I^{2*} \times \text{AIR}_g$), as described previously (29, 30) (SAAMII Institute, Seattle, WA). S_I^{2*} explains the effects of insulin on glucose disposal rates while S_G^{2*} quantifies the effects of glucose to cause its own transport via mass action at basal insulin concentrations.

Muscle Analysis

Immediately post collection, muscle samples were washed in ice-cold saline, with visible fat removed before being frozen in liquid nitrogen and transferred to -80°C until analysis. Muscle tissue homogenates were used for Western Blot Protein analysis. Protein content of the homogenates was quantified using Lowry's method (Bio-Rad DC protein assay) with 20 μg of total protein separated using 7.5% precast polyacrylamide gels before being transferred using a semi-dry method to nitrocellulose membranes (Bio-Rad).

Immunoblotting

Membranes were blocked in 5 % BSA (1 hour), and polyclonal antibodies pAkt308, pAkt473, total Akt, AS160, pAS160 on Thr642 (Cell Signalling) and IP6K1 (Abcam) incubated overnight at 1:1,000 in 5 % BSA at 4°C . Membranes were then washed and incubated with anti-rabbit secondary antibody (Cell Signalling; 1:10,000) in 0.5 – 5 % BSA. Membranes were quantified using Odyssey[®] Fc Imaging System (LI-COR). Blots were normalized to total protein (31) as this method shows greater sensitivity than 'house-keeping' proteins.

Real-time quantitative PCR

Total RNA from muscle biopsy samples (20 mg) was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the standard manufacturers protocol. Cell lysates were homogenized using Qias shredder spin columns (Qiagen, Hilden, Germany). The concentration and purity of extracted RNA were measured at 260 nm by spectrophotometry using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Extracted RNA samples were stored at -80°C . For relative quantification of mRNAs, total RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, up to 1 μg RNA was reverse transcribed to cDNA in a final volume of 20 μl using Oligo (dT)₁₅ primers (0.5 μg /reaction). Each quantitative real-time PCR reaction mixture (20 μl) contained 1 μl of RT product (cDNA transcribed from 1 μg of total RNA) was performed using Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The mixture was initially incubated at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 52°C for 15 s and 72°C for 30 s. PCR reactions were carried out on a Rotor-Gene Q (Qiagen, Hilden, Germany) in triplicate. Samples were normalized relative to the mRNA level of GAPDH. For each individual, all samples were simultaneously analyzed in one assay run. Measurements of the relative distribution of each target gene were performed for each individual; a cycle threshold (C_T) value was obtained by subtracting GAPDH C_T

values from respective target C_T values. The expression of each target was then evaluated by the Rotor-Gene Q Software 2.3 (Qiagen, Hilden, Germany).

In vitro experiment

Myoblasts from the muscle-derived mouse C2C12 cell line (ATCC # CRL - 1772) were grown in growth media (GM) of Dulbecco's modified Eagle's Media (DMEM; Gibco # 22320), supplemented with 10 % FBS, penicillin (50 U.mL^{-1}) and streptomycin (50 U.mL^{-1}) in a standard manner (37°C , 5 % CO_2 , 100 % humidity) until $\approx 80\%$ confluent. Cells were trypsinized and seeded for experimental conditions in standard 6-well. To induce differentiation into myotubes confluent cells were washed in Dulbecco's PBS (DPBS) and incubated in differentiation media (DM) of DMEM with 2 % equine serum, penicillin (50 U.mL^{-1}) and streptomycin (50 U.mL^{-1}) for 96 hours with DM changed every 24 hr before experimental conditions were applied.

After 96 hr for myotube formation, cultures were incubated under control, hyperglycaemic and hyperinsulinaemic conditions \pm N2-(m-Trifluorobenzyl), N6-(p-nitrobenzyl)purine (TNP), a pan-IP6K inhibitor. For hyperinsulinaemic treatment, myotubes were incubated in 100 nM insulin (Sigma, UK) for 24 hr in serum-free DMEM containing 5mM glucose (32) with control cells incubated in DMEM containing 5mM glucose for the same time period. For hyperglycaemia, C2C12 cells were treated in serum-free DMEM containing 30mM D-glucose for 24hr (33). Each treatment was performed \pm TNP at $10\mu\text{M}$ (34). At the 24 hr point, total protein was extracted. Cells were aspirated and washed on ice with ice cold PBS before 400 μL lysis buffer with protease inhibitor (1:100) (Cell Signalling) was added. After 20 minutes incubation, cells were scrapped into 1.5ml Eppendorf tubes, spun (6 min, 6000 rpm) and supernatant removed and aliquoted for protein quantification (Lowry, Bio-Rad DC protein assay) and later analysis of proteins of interest by Western blot (described above).

Statistics

The area under the curve for both glucose (AUC_{Glu}) and insulin (AUC_{Ins}) were calculated using the trapezoidal rule. Differences over time and between conditions were evaluated by two-way repeated measures analysis of variance. Tukey's *post hoc* tests were used when statistical significance was found. Linear regression analyses were carried out to test for significance where appropriate. All statistical tests were carried out using the statistical software package SPSS (version 15). Data are expressed as mean (SE). Statistical significance was set at the level $P < 0.05$.

Results

Correlation analysis

IP6K1 has been shown in cell culture and animal models to be implicated in reduced glucose control. One of the key aims of his research was to assess if muscle and plasma IP6K1 correlated with whole body measures of glucose control. Figure 1A & B show correlation analysis between plasma IP6K1 concentration and two-compartment measures of peripheral glucose control. This data has been combined with that of previously published work to include a range of insulin resistant individuals including type 2 diabetics (26). Neither S_I^{2*} ($r = 0.402$; $P = 0.055$) or S_G^{2*} ($r = 0.151$; $P = 0.281$) showed significant relationship with plasma IP6K1. Baseline measures HbA1c ($r = 0.357$; $P = 0.080$), fasting blood glucose ($r = 0.232$; $P = 0.185$), fasting insulin ($r = 0.365$; $P = 0.075$), % body fat ($r = 0.028$; $P = 0.457$) and $\text{HOMA}_{\beta\text{-Cell}}$ ($r = 0.006$; $P = 0.491$) were also correlated with plasma IP6K1 with only HOMA_{IR} demonstrating a significant relationship ($r = 0.429$; $P = 0.043$) with this measure. A

full set of correlation a data is displayed in Table 1. No relationship was noted between muscle IP6K1 protein content and S_I^{2*} ($r = 0.019$; $P = 0.961$). This comparison was for the current data set (Figure 1C) as muscle tissue was not collect in our earlier work (26).

Exercise Intervention

Immediately following the labeled intravenous glucose load, AUC_{Glu} was significant lower post intermittent ($P = 0.008$) and continuous ($P = 0.016$) exercise treatments when compared to the resting control trial. No difference was noted for AUC_{Glu} between exercise treatments ($P = 0.084$) (Figure 2D). Despite a trend for being lower post treatment, neither exercise condition affected AUC_{Ins} (Figure 2E; $P = 0.421$). Endogenous glucose, labeled glucose and insulin concentrations were modeled to determined two-compartment measures of S_I^{2*} , S_G^{2*} and HGP. Both exercise conditions demonstrated a significant increase in S_I^{2*} over the control trial ($P < 0.001$). S_I^{2*} was also significantly higher following high-intensity intermittent when compared to traditional moderate intensity exercise (Figure 2A; $P < 0.01$). Despite being higher following both exercise conditions, S_G^{2*} was not found to be statistically different from resting control (Figure 2B; $P = 0.561$)

Skeletal muscle Signaling

Comparisons between treatments in human skeletal muscle samples were made as fold change from fasting control. IP6K1 was significantly lower immediately post intermittent exercise compared to fasted samples ($P = 0.001$) with no difference noted for the same comparisons for continuous exercise ($P = 0.337$; Figure 3A). Phosphorylation of Akt at serine 308 was elevated for intermittent exercise ($P = 0.003$) above fasted values, with no difference for the same comparison with the continuous treatment ($P = 0.175$; Figure 3B). There was no difference between treatments for $pAkt^{473}$ ($P = 0.200$; Figure 3C). The downstream target for Akt, AS160 was significantly increased following both intermittent ($P = 0.012$) and continuous exercise ($P = 0.041$; Figure 3D). Intramuscular IP6K1 mRNA expression decreased significantly after both exercise treatments ($P < 0.01$) with continuous exercise lower than the intermittent protocol ($P < 0.05$). Akt and GLUT4 expression was significantly higher following intermittent and continuous exercise when compared to fasting samples ($P < 0.01$). PDK1 mRNA expression was significantly greater for the intermittent exercise treatment only ($P < 0.01$).

Insulin signaling in C2C12 cell

C2C12 skeletal muscle cell were treated with both insulin and glucose +/- TNP (10 μ M) to assess the effects of the stated treatment on IP6K1. Insulin treatment increased IP6K1 protein content over the control treatment ($P = 0.010$). Insulin + TNP treatment was not different to control ($P = 0.647$) but was significantly lower compared to the insulin condition ($P = 0.008$). Glucose treatment increased IP6K1 ($P = 0.007$) which was lowered with the addition of TNP (Glu + TNP; $P = 0.008$). Phosphorylation of Akt at 308 was significantly reduced with insulin when compared to the control condition ($P = 0.041$) and elevated in the insulin + TNP over the insulin only treatment ($P = 0.030$). Twenty-four hours of insulin treatment with and without TNP increased p/t Akt⁴⁷³ in the C2C12 skeletal muscle cells ($P < 0.05$). In addition, p/t Akt⁴⁷³ was significantly higher in the insulin only trial when compared with insulin + TNP ($P < 0.05$). The 160 kDa Akt substrate (AS160), one of the last proximal steps in glucose transport in skeletal muscle was elevated in both the insulin and insulin + TNP treatments ($P < 0.05$). The same target was significantly reduced with the addition of glucose + TNP to the treatment media (Figure 5D; $P = 0.039$).

Discussion

Previous work has shown that IP6K1 KO mice display both normal glycaemic control and low circulating plasma insulin (13). In addition, IP6K1 gene deleted mouse embryonic

fibroblasts (MEFs) demonstrate increased Akt phosphorylation and glucose transport rates (13, 11). While important, these basic and reductionists approaches lack complete translational relevance. Here we show that IP6K1 protein content in insulin resistant skeletal muscle does not correlate with whole body measures of glucose control. Conversely, exercise decreased IP6K1 protein content in human skeletal muscle and that the exercise treatment that caused the greatest improvements in S_I^{2*} also caused the greatest decrease in muscle IP6K1 content. A supplementary aim of this work was to investigate IP6K1 roles in insulin signalling in skeletal muscle. Our work showed that the chemical inhibition of IP6K1 *in vitro* increased phosphorylation of Akt at both Ser⁴⁷³ and Thr³⁰⁸ in the skeletal muscle C2C12 cell line. While insulin increased AS160^{Thr642}, IP6K1 inhibition had no additive effect on this important target in insulin-stimulated glucose uptake suggesting that IP6K1 may not interfere with AS160 activity despite increasing its upstream activator pAkt.

The production of IP7, stimulated in part by insulin, is known to compete with PIP3 at the PH domain of Akt, inhibiting subsequent translocation and phosphorylation of Akt by PDK1. This process may represent a negative feedback mechanism whereby hyperinsulinaemia eventually decreases Akt activity (11). Given this notion, it was hypothesised that muscle IP6K1 protein content would correlate with two-compartment modules of (S_I^{2*}), yet despite a negative relationship, this was not found to be significant ($r = 0.019$; $P = 0.961$). Interestingly, plasma IP6K1 demonstrated the strongest relationship with S_I^{2*} , although not significant ($r=0.389$; $P = 0.110$). This would suggest that muscle and plasma IP6K1 are not key mediators in the development of whole body insulin resistance, despite being seemingly important in an *in vitro* model of insulin resistance. It's worth noting the relatively small sample size in the current work and that this study focused on skeletal muscle in isolation. Thus future work should examine the role of IP6K1 in adipose tissue as well as other insulin sensitive tissue. In addition to this, a major limitation of this data is the assumption that muscle protein signalling in the vastus lateralis is reflective of insulin signalling in other muscle groups and insulin sensitive tissue. Data elsewhere reports that glucose uptake is different for different muscle types (35). Our data did however show a significant relationship between HOMA_{IR} and plasma IP6K1 with the former known to correlate with one compartment modules of insulin sensitivity (36) and validated against the euglycaemic-hyperinsulinemic clamp technique (37), considered the gold standard assessment of insulin sensitivity and secretion (38, 39). Taken together, this suggests that HOMA_{IR} is a useful measure in the assessment of glucose control and that circulating IP6K1 may be implicated as one of the available predictor of insulin resistance.

In skeletal muscle, insulin-mediated IRS activation causes the downstream phosphorylation of Akt and AS160 to facilitate translocation of GLUT4 proteins to the plasma membrane, where they fuse, leading to increased glucose uptake into the cell (40). Phosphorylation of AS160^{Thr642} was also elevated post both exercise conditions suggesting that exercise can increase S_I^{2*} and pAS160^{Thr642} while also decreasing muscle IP6K1. Our data does not allow us to speculate on a possible mechanism linking IP6K1 and AS160. Yet it is likely that any relief on the inhibitory effects of IP6K1 on Akt (11) would likely result in an increase in pAS160^{Thr642}, particularly in a post-exercise muscle cellular environment. Chemical inhibition of IP6K1 with TNP *in vitro* elevated Akt³⁰⁸ and Akt⁴⁷³ phosphorylation yet had no additive effect over insulin on pAS160^{Thr642}, suggesting that IP6K1 may play a part in Akt activity but this may not be sufficient to change downstream related targets.

Contrary to our hypothesis, IP6K1 protein content was not correlated with whole body measures of S_I^{2*} or S_G^{2*} . At least from the current data set, it appears that exercise can decrease muscle IP6K1 content in the acute period (~1.5 hr) (41) following muscle contraction and that the same stimulus improved S_I^{2*} . We note two novel findings within our exercise data 1) high-intensity exercise had a greater effect on two-compartment models of

insulin sensitivity when compared to the lower intensity exercise at 90% of lactate threshold and 2) high-intensity exercise decreased hepatic glucose production (HGP) in the 4 hours following exercise. Continuous moderate-intensity exercise offered clear improvements in S_I^{2*} but showed no change in HGP. This data suggests that greater improvements in glucose control can be obtained with high-intensity exercise over more traditional forms in pre-diabetic individuals. The cellular mechanisms explaining improvements in insulin sensitivity in response to exercise have been well documented and reviewed elsewhere (42, 43). The mechanisms by which muscle contraction influences other insulin sensitive tissue remains a key question. Elevated HGP is a major contributing factor to hyperglycemia in type 2 diabetes (44) owing to hepatic insulin resistance. Increased glucose Ra is a normal and well documented response to exercise and that higher intensity exercise is met with a greater glucose Ra over moderate intensity exercise (45, 46, 47). The increase in glucose Ra during exercise is a product of increased hepatic AMP, elevated AMPK levels (48), increased hepatic glucagon delivery (49) and sensitivity (50). Indeed, increased glucagon and reduced insulin (51, 52, 53) are key contributory factors of HGP during exercise. The rise in glucagon causes a decrease in hepatic glycogenolysis and gluconeogenesis, while a reduction in insulin secretion also causes hepatic glycogenolysis (52). Thus post exercise hepatic Ra is likely to be down-regulated following higher-intensity exercise due to a reduction in hepatic insulin requirements (54), resynthesis of liver glycogen (55) and an exercise induced increase in hepatic AMPK (56) and IRS-2 (57). AMPK inhibits phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (58), both of which are key enzymes responsible for reducing gluconeogenesis while upregulation of IRS-2 is associated with improved hepatic sensitivity to insulin (59). Data shows that post exercise ingestion of ^{13}C -glucose increased hepatic glycogen resynthesis by $0.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ over a 4 hr period in humans (60, 61).

High-intensity exercise also decreased muscle IP6K1 content while increasing $\text{pAkt}^{\text{Thr308}}$. Historical exercise data consistently shows increased Akt expression post exercise due to its key role in both protein synthesis (62) and insulin-stimulated glucose uptake (12). Short-term TNP treatment increases $\text{pAkt}^{\text{Thr308}}$, $\text{pGSK}\alpha^{\text{Ser21}}$ and $\text{pGSK}\beta^{\text{Ser9}}$ in mice (63), suggesting that inhibition of IP6K1 has the potential to increase the activity of key proteins in the insulin signal cascade. This notion supported by the current study (Figure 5F). The mechanism by which high-intensity exercise decreases IP6K1 is currently unexplained. Previous research has suggested that increased intercellular Ca^{2+} levels (64) may interfere with IP6K1-IP7 signalling (65). Muscle contraction requires the depolarisation of the sarcolemma resulting in Ca^{2+} releases from the muscle sarcoplasmic reticulum. Thus the increase in intracellular Ca^{2+} concentration maybe the link between exercise and reduced IP6K1 levels. Yet IP₆ has been shown to suppress excitatory neurotransmission in *hippocampal neuron* by inhibiting the presynaptic Syt1–C2B domain (66). The synaptotagmin 1 (Syt1) is a key Ca^{2+} sensor essential for synaptic membrane fusion. The interaction of IP6K1 and its products on Ca^{2+} actions in skeletal muscle requires further investigation.

Ghoshal et al. (63) suggested that IP6K1 inhibition in rodents may reduce the inhibitory effects of IP7 on both pAkt and energy expenditure, the latter caused in an AMPK dependent mechanism. IP6K/5-IP7 inhibits Akt and LKB1-AMPK pathways (67, 68, 69, 70) with both pathways known to enhance UCP1 mediated thermogenesis (59, 71, 72, 73, 74). Exercise also stimulates AMPK during muscle contraction with elevated pAkt-GSK3 a current picture in a post exercise muscle environment. Thus, IP6K1 mediated regulation of AMPK- and Akt-dependant mechanisms has the potential to up-regulate glucose transport and offer the appearance of improved whole body insulin sensitivity. In support of this former point, data from our laboratory shows that $\text{pAMPK}^{\text{Thr172}}$ is increased in C2C12 muscle cells in response to insulin and insulin-like growth factor treatment when supplemented with TNP (Figure 5F).

AMPK protein content was not determined in human muscle homogenate from the current study.

In conclusion, muscle IP6K1 did not correlate with insulin sensitivity, as measured by the labelled IVGTT. However, plasma IP6K1 was related to HOMA_{IR} in hyperinsulinaemic pre-diabetic humans suggesting that global IP6K1 and not muscle bound IP6K1 maybe implicated in insulin resistance. High-intensity exercise did however reduce muscle IP6K1 content and this is met with a significant increase in insulin sensitivity. Here we have shown that TNP inhibits IP6K1 in C2C12 myotubes, but this is not accompanied with changes in AS160 phosphorylation. Taken together, these data suggest that muscle IP6K1 may play a part in insulin resistance but do not provide a complete and whole picture with other signalling intermediates likely to be involved.

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Figure 1. Correlation analysis between plasma IPK61, glucose effectiveness (S_G^{2*} ; $r = 0.151$; $P = 0.281$), insulin sensitivity (S_I^{2*} ; $r = 0.402$; $P = 0.055$) and homeostasis model of insulin resistance ($HOMA_{IR}$; $r = 0.429$; $P = 0.043$). Figure 1A, 1B, 1C include additional data from a previously published paper (Mackenzie *et al.*, 2011) and the current data set ($n = 17$). Correlation analysis includes both pre-diabetics and type 2 diabetics. Figure 1D shows correlation analysis for muscle IP6K1 protein content ($n = 9$) with S_I^{2*} from the current data only.

Figure 2. Insulin Sensitivity (S_I^{2*}) (A), glucose effectiveness (S_G^{2*}) (B) and Hepatic Glucose Production (HGP) (C) in response to a control, continuous and intermittent exercise trials.

The integrated area under the curve for arterialized blood glucose (D) and plasma insulin (E) following iv labeled glucose loads 4 hr post trials. *Denotes significant difference between resting control ($P < 0.01$). † Denotes difference between continuous and intermittent exercise ($P < 0.001$)

Figure 3. Muscle protein content of IP6K1 (A), phosphorylation of Akt at Thr308 (B), Akt at Ser473 (C), AS160 at Thr642 (D) and representative blots (E) (n=9). * $P < 0.05$ vs. Fasting (pre-exercise).

Figure 4. Intramuscular mRNA expression of IP6K1 (A); Akt (B); GLUT 4 (C); PDK1 (D). Values are expressed as fold change from fasting pre-exercise and post each trial as mean (SE) (n=9). *Different from Fast, $P < 0.01$; ‡ different to Rest, $P < 0.05$; †different to continuous exercise, $P < 0.05$.

Figure 5. Muscle protein content of IP6K1 (A), phosphorylation of Akt at Thr308 (B), Akt at Ser473 (C), AS160 at Thr642 (D) and representative blots (E) for C2C12 treatments. * $P < 0.05$ vs. control. α $P < 0.05$ vs. Ins+TNP and † $P < 0.05$ vs. Ins. Data are mean \pm SE (n=4). Figure 5F shows PathScan® Akt Signaling Antibody Fluorescent read (700). * $P < 0.05$ vs. control, † $P < 0.05$ vs. IGF, α $P < 0.05$ vs. Ins and γ $P < 0.05$ vs. Ins+TNP. Data are mean \pm SE (n=4).

Table 1. Correlation analysis between plasma IP6K1 and measures of glycemic control

	Pre-Diabetics	Type 2 Diabetics	Combined
S_I^{2*}	$r = 0.033$ (0.932)	$r = 0.733$ (0.025)*	$r = 0.402$ (0.055)
S_G^{2*}	$r = 0.247$ (0.521)	$r = 0.472$ (0.200)	$r = 0.151$ (0.281)
HbA1c	$r = 0.194$ (0.595)	$r = 0.310$ (0.493)	$r = 0.357$ (0.080)
Fasting blood glucose	$r = 0.119$ (0.760)	$r = 0.043$ (0.913)	$r = 0.232$ (0.185)
Plasma Fasting insulin	$r = 0.179$ (0.645)	$r = 0.340$ (0.370)	$r = 0.365$ (0.075)
% body fat	$r = 0.086$ (0.825)	$r = 0.061$ (0.870)	$r = 0.028$ (0.457)
HOMA $_{\beta\text{-Cell}}$	$r = 0.171$ (0.661)	$r = 0.261$ (0.498)	$r = 0.006$ (0.491)
HOMA $_{IR}$	$r = 0.194$ (0.617)	$r = 0.311$ (0.415)	$r = 0.429$ (0.043)*

Values are means (SEM). Insulin sensitivity (S_I^{2*}); glucose effectiveness (S_G^{2*}); Body Mass Index (BMI); Glycosylated Haemoglobin (HbA1c), Homeostasis Model Assessment of Insulin Resistance (HOMA $_{IR}$); β -Cell function (HOMA $_{\beta\text{-Cell}}$).









